***Zobellia galactanovorans* gen. nov., sp. nov., a marine species of *Flavobacteriaceae* isolated from a red alga, and classification of [*Cytophaga*] *uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Zobellia uliginosa* gen. nov., comb. nov.**

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A mesophilic, aerobic, non-flagellated, gliding bacterium, forming yellow colonies and designated Dsij^T, was isolated from a red alga on the sea-shore of Roscoff, Brittany, France. Dsij^T was selected for its ability to actively degrade both agars and carrageenans. The Gram-negative cells occurred singly or in pairs as long rods. The temperature range for growth was 13–45 °C, with an optimum at 35 °C. The pH range for growth at 35 °C was from 6.0 to 8.5, with an optimum around pH 7.0. The NaCl concentrations required for growth at 35 °C and pH 7.0 ranged from 5 to 60 g l⁻¹, with an optimum around 25 g l⁻¹. The G+C content of the genomic DNA was 42–43 mol %. Phylogenetic analysis of 16S rRNA gene sequences indicated that strain Dsij^T is closely related to [*Cytophaga*] *uliginosa* DSM 2061^T. Phenotypic features, however, allowed Dsij^T and [*Cytophaga*] *uliginosa* strains to be distinguished on the basis of ten traits (spreading behaviour, assimilation of eight compounds and amylase production). Their total protein profiles were also different and DNA–DNA hybridization experiments confirmed that Dsij^T constitutes a new species, distinct from [*Cytophaga*] *uliginosa*. Based on the phenotypic features and the phylogenetic relationships of the *Flavobacteriaceae*, a new genus designated *Zobellia* gen. nov. is proposed to include *Zobellia galactanovorans* gen. nov., sp. nov., while [*Cytophaga*] *uliginosa* becomes *Zobellia uliginosa* comb. nov. The type strain of *Zobellia galactanovorans* is Dsij^T (= DSM 12802^T = CIP 106680^T).

Keywords: *Zobellia galactanovorans*, *Zobellia uliginosa*, [*Cytophaga*] *uliginosa*, *Flavobacteriaceae*, carrageenase and agarase activities

INTRODUCTION

Agars and carrageenans are cell wall galactans extracted from various marine red algae. They exhibit unique rheological properties and are widely used as

texturing agents in various industries (De Ruiter & Rudolph, 1997). They consist of a linear backbone of D-galactose residues linked by alternating $\alpha(1 \rightarrow 3)$ and $\beta(1 \rightarrow 4)$ linkages. In agars the $\alpha(1,4)$ -linked galactose units are in the L configuration whereas they are in the D configuration in carrageenans. A further layer of complexity is the occurrence of a 3,6-anhydro bridge in the $\alpha(1,4)$ -linked galactose residues and the number of ester-sulfate substituents per digalactose repeating unit, that vary from 0 in agarose to 3 in λ -carrageenan (Rees, 1969; Craigie, 1990).

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The GenBank accession number for the 16S rDNA sequence of strain Dsij^T is AF208293.

A bacterium that degrades microbiological agar was first discovered at the beginning of this century (Gran, 1902). Interestingly, as early as the 1940s, Humm (1946) discriminated between marine bacteria according to their specific hydrolytic activity on gel-forming extracts from seaweeds that were later distinguished as agarophytes and carrageenophytes when the structures of agars and carrageenans became known (Araki & Arai, 1956, 1957; Rees, 1962). Since then, many agarolytic and carrageenolytic activities have been described from *Bacteria* as diverse as *Flavobacterium* (ZoBell & Upham, 1944), *Cytophaga* (Duckworth & Turvey, 1969; Sarwar *et al.*, 1983; Potin *et al.*, 1991), *Pseudomonas* (Weigl & Yaphe, 1966; Hofsten & Malmqvist, 1975; Vattuone *et al.*, 1975; Greer & Yaphe, 1984), *Pseudoalteromonas* and *Alteromonas* (Groleau & Yaphe, 1977; Morrice *et al.*, 1983; Leon *et al.*, 1992; Potin *et al.*, 1993), as well as *Streptomyces* (Bibb *et al.*, 1987) and *Vibrio* (Aoki *et al.*, 1990; Sugano *et al.*, 1993).

Very little information, however, is available on the carrageenase activities of the *Flavobacteriaceae* and *Cytophagaceae* (Sarwar *et al.*, 1983; Holmes *et al.*, 1984; Reichenbach, 1989; Bernardet *et al.*, 1996). The marine bacterium '[*Cytophaga*] *drobachiensis*' strain Dsij^T (square brackets indicate a generically misnamed taxon and quotation marks indicate a name not yet validated), isolated from the red alga *Delesseria sanguinea*, was shown to possess the enzymic machinery to completely degrade various red algal galactans, including κ -carrageenase (Potin *et al.*, 1991; Barbeyron *et al.*, 1998), ι -carrageenase (Potin *et al.*, 1991, 1992; Barbeyron *et al.*, 2000) and two different β -agarases (Potin, 1992). Humm (1946) had described the behaviour of a non-motile marine pseudomonad, '*Pseudomonas drobachienae*' (Lundestad) Stanier (1941), on gels prepared from the red algal genera *Chondrus*, *Agardhiella* and *Gracilaria*, which contain κ -carrageenan, ι -carrageenan and agar, respectively, as main cell wall polysaccharides. The morphological and biochemical features reported by Humm (1946) for '*P. drobachienae*' are very similar to the characteristics of strain Dsij^T (Potin, 1992). However, Potin (1992) showed that this latter strain behaves as a gliding bacterium. Based on the above results, on the definition of the genus *Pseudomonas*, which includes species motile by flagella, on the report of '*P. drobachienae*' as *incertae sedis* (Doudoroff & Palleroni, 1974) as well as on the evidence showing that strain Dsij^T belongs to the *Cytophaga*-*Flavobacterium*-*Bacteroides* (CFB) group, the name '[*Cytophaga*] *drobachiensis*' (Lundestad) was proposed for strain Dsij^T in our reports on the cloning of its carrageenase genes (Barbeyron, 1993; Flament, 1999; Barbeyron *et al.*, 1998).

Since the studies of Bernardet *et al.* (1996) and Nakagawa & Yamasato (1993, 1996), the order *Cytophagales*, i.e., the rRNA superfamily V of the *Bacteria* or the CFB group (Bernardet *et al.*, 1996), has been revisited. The family *Flavobacteriaceae* has been

amended (Bernardet *et al.*, 1996) and it now includes *Flavobacterium*, *Capnocytophaga* (Leadbetter *et al.*, 1979) and *Chryseobacterium* as major genera (Vandamme *et al.*, 1994). The type genus of the family, *Flavobacterium*, has also been amended (Bernardet *et al.*, 1996). However, misnamed taxa such as those of the [*Flexibacter*] *maritimus* group, i.e. [*Flexibacter*] *maritimus* and [*Flexibacter*] *ovolyticus* (Bowman *et al.*, 1998), as well as [*Cytophaga*] *uliginosa*, [*Cytophaga*] *marinoflava*, [*Cytophaga*] *latercula* and the *Melosira*-colonizing bacterium strain IC166 (Bowman *et al.*, 1998), are classified in this family.

In the present work, we describe in detail the strain Dsij^T, so far known as '[*Cytophaga*] *drobachiensis*'. Several lines of evidence, based on the sequence of its 16S rDNA, DNA-DNA hybridizations, phylogenetic analyses and phenotypic features, indicate that this taxon is very closely related to [*Cytophaga*] *uliginosa*. It is thus proposed that these two species represent a novel taxon, referred to as *Zobellia* gen. nov. '[*Cytophaga*] *drobachiensis*' thus becomes *Zobellia galactanovorans* strain Dsij^T gen. nov., sp. nov., while [*Cytophaga*] *uliginosa* is renamed *Zobellia uliginosa* comb. nov.

METHODS

Enrichment, isolation and growth conditions. '[*Cytophaga*] *drobachiensis*' strain Dsij^T was isolated from the red alga *Delesseria sanguinea* (Huds.) Lamour. (Ceramiales, Rhodophyta) (Potin *et al.*, 1991). Algae were collected in the English Channel near Roscoff (Brittany, France) and thallus fragments were deposited on a basal salts medium (Quatrano & Caldwell, 1978), which contained 2% (w/v) ι -carrageenan. To obtain pure cultures with galactanolytic activities, subcultures of isolates were streaked on the same medium supplemented with 2% ι -carrageenan, 1.5% (w/v) agar, 1% κ -carrageenan or 1.2% λ -carrageenan mixed with 1.5% agar. Isolated strains were grown in ZoBell medium 2216E (ZoBell, 1941), liquid or solidified with agar or carrageenan. When it was desirable to avoid attack of the substratum, strains were grown on ZoBell solidified with 0.7% (w/v) Phytagel (a gellan gum; Sigma). Stock cultures of isolate Dsij^T were stored in the culture medium at 4 °C. For long-term storage, pure cultures were stored at -80 °C in the same medium containing 20% (v/v) glycerol. [*Cytophaga*] *uliginosa* DSM 2061^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) collection, Braunschweig, Germany. All of the *Cellulophaga* species, namely *Cellulophaga baltica* (LMG 18535^T), *Cellulophaga fucicola* (LMG 18536^T) and *Cellulophaga lytica* (ATCC 23178^T), were generously provided by Dr J.-F. Bernardet. These marine bacteria were grown in ZoBell medium.

Determination of growth conditions. Growth was monitored by measuring the increase in optical density at 600 nm using a Spectronic 20D spectrophotometer (Bioblock). All growth experiments were performed in duplicate. The optimal pH value was determined in the *Cytophaga* marine medium described by DSMZ, composed of (l⁻¹) 1 g tryptone, 1 g yeast extract, 0.7 g KCl, 6.3 g MgSO₄·7H₂O, 4.6 g MgCl₂·6H₂O, 1.2 g CaCl₂·2H₂O, and using 10 mM MES for pH 5.0, 5.5 and 6.0, 10 mM PIPES buffer for pH 6.5 and 7.0,

10 mM HEPES buffer for pH 7.5, 10 mM Tris/HCl for pH 8.0 and 8.5, and no buffer for pH 9.0. The effect of NaCl on growth was determined in the same medium containing 0, 1, 2, 3, 4, 5, 6, 7 or 8% (w/v) NaCl. The temperature range for growth was determined between 10 and 50 °C. The effects of pH and NaCl concentration were determined at 30 °C.

Phenotypic characterization. Morphological features were investigated with cells in exponential phase and grown in *Cytophaga* marine medium. Gliding motility was tested with colonies on Phytigel, mounted between a slide and a cover glass, by phase-contrast microscopic observation of the living cells present at the edges of the colony. To determine the respiratory type, bacteria were inoculated into Veillon tubes containing ZoBell medium solidified with 0.6% agar. Oxygen was removed from the medium by boiling. To determine their oxidative or fermentative behaviour, bacteria were inoculated into a modified Leifson O-F medium (Hugh & Leifson, 1953; Smibert & Krieg, 1981), containing 0.5% (w/v) glucose. Oxidase activity was assayed with disks impregnated with dimethyl-*p*-phenylenediamine oxalate (Diagnostics Pasteur). Catalase activity was assayed by mixing one colony from a ZoBell agar plate with a drop of hydrogen peroxide (10%, v/v). The ability to use simple carbohydrates as carbon sources was tested in Basal Salt Medium (Quatrano & Caldwell, 1978), containing the carbohydrate under investigation at a concentration of 0.5% (w/v). The strain was assayed for amylase activity using starch at a concentration of 0.2% (w/v) in ZoBell agar or in ZoBell Phytigel plates. DNase activity was detected in DNA agar (Diagnostics Pasteur), supplemented with 25 g NaCl l⁻¹. Amylase and DNase activities were revealed with a lugol solution on starch agar and with 1 M HCl on DNase agar, respectively. Cellulolytic activities were tested using strips of Whatman no. 1 or no. 3 MM Chr filter paper, on solid medium, as described by Dungan *et al.* (1989), and in liquid medium, either in the presence or in the absence of carbon sources, according to Smibert & Krieg (1981). Paper strips were sterilized by UV irradiation and placed in the tubes so that a portion of the strips extended above the level of the medium. Plates and tubes were incubated at 25 °C for 1 month. Liquefaction of CM-cellulose was tested after incubation of a dense inoculum in tubes filled with ZoBell medium supplemented with 3% (w/v) CM-cellulose for 15 d at 25 °C. Chitinolytic activity was tested as described by Dungan *et al.* (1989). Agarase, κ -carrageenase and ι -carrageenase activities were tested both by a reducing sugar assay (Kidby & Davidson, 1973) and by testing for degradation of a ZoBell medium solidified with 15 g agar l⁻¹, 10 g κ -carrageenan l⁻¹ or 20 g ι -carrageenan l⁻¹. Strains which made a hole in the substratum were considered as positive. Production of flexirubin was assessed by flooding a 4 d plate culture with 20% (w/v) potassium hydroxide followed by the observation of changes in colony colour from yellow to red or brown (Reichenbach *et al.*, 1974). Other tests were performed by utilization of API 20 NE, API 50 CH and API ZYM strips (BioMérieux) and by utilization of BIOLOG GN microplates. For these tests, the suspending medium was adjusted to a NaCl concentration of 25 g l⁻¹ and the preparations were incubated at 30 °C for 1 week.

Antibiotic susceptibility. Sensitivity to chloramphenicol (10 µg ml⁻¹), penicillin G (200 µg ml⁻¹), streptomycin (200 µg ml⁻¹), kanamycin (200 µg ml⁻¹), ampicillin (200 µg ml⁻¹), rifampicin (10 µg ml⁻¹) and tetracycline (10 µg ml⁻¹) (all from Sigma) was tested at 30 °C.

Light and electron microscopy. An Olympus BH2 microscope

equipped with an Olympus OM-2 camera was used to observe the bacteria and to obtain photomicrographs. Gram staining was carried out as described by Conn *et al.* (1957). For negative staining, 20 µl of a bacterial suspension fixed with 2% (w/v) glutaraldehyde was dropped onto Formvar/carbon-coated grids (400 mesh) and stained with 4% (w/v) uranyl acetate. Micrographs were taken on a model CM100 electron microscope (Philips) with an acceleration voltage of 80 kV.

Isolation of DNA. Genomic DNAs were obtained by using the procedure described by Barbeyron *et al.* (1984). The DNA was purified on a caesium chloride gradient and purity was checked spectrophotometrically.

DNA base composition. The Chargaff's coefficient of the DNA, expressed as the molar percentage of guanine plus cytosine (mol% G+C), was determined by the spectroscopic method of Ulitzur (1972) using *Escherichia coli* DNA as a standard, and also by measuring the melting points (Marmur & Doty, 1962), using a Kontron spectrophotometer model Uvikon 940 equipped with a system disk 9009 (Kontron Instruments). The spectrophotometer was equipped with a Huber cryothermostat (Ministat HS40), which was regulated by a Huber temperature programmer (model PD415; Bioblock). Standard DNAs from *E. coli* (50 mol% G+C), *Clostridium perfringens* (26.5 mol% G+C) and *Micrococcus luteus* (72 mol% G+C), were purchased from Sigma.

Small subunit rDNA sequencing. 16S rDNA was amplified by PCR with *Taq* polymerase (Promega), using the genomic DNA from strain Dsij^T as template and two primers specific for *Bacteria* [8F primer: AGAGTTTGATCCTGGCTCAG (Hicks *et al.*, 1992) and 1492R primer: GGTTACCTGTTACGACTT (Kane *et al.*, 1993)]. PCR reactions were typically carried out in a volume of 50 µl containing 50–100 ng template, 100 ng of each of the two specific primers, 250 µM of each dNTP, 1.5 mM MgCl₂, 1 × buffer (Promega) and 2.5 U polymerase. The different steps of PCR were as follows: 5 min at 95 °C; then 25 cycles of 1.5 min at 95 °C, 1.5 min at 53 °C and 2.5 min at 72 °C; then finally a polymerization step of 8 min at 72 °C. PCR products were cloned in vector pCRII2.1 and sequenced, using Texas-red labelled primers, a Thermosequenase kit (RPN 2444; Amersham) and a Vistra 725 automated sequencer.

Phylogenetic analysis of the rDNA gene sequence of strain Dsij^T. 16S rDNA sequences were aligned manually with a representative set of 16S rRNA sequences obtained from the Ribosomal Database Project (Maidak *et al.*, 1999) and from recent GenBank releases (Benson *et al.*, 1999). The secondary structure was used as a guide to ensure that only homologous regions were compared. Of the 1483 nucleotides that were sequenced, 1257 were used in the phylogenetic analysis. Distance matrix, maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods were applied, as implemented in the ARB software package (Ludwig & Strunk, 1997). The neighbour-joining method (Saitou & Nei, 1987) was performed with the Jukes and Cantor distance correction (Jukes & Cantor, 1969) and bootstrap analysis was used to provide confidence estimates for the phylogenetic tree topologies (Felsenstein, 1985).

DNA–DNA hybridization. Genetic relatedness was investigated by slot-blot DNA–DNA hybridization, using the ECF random-prime labelling and signal amplification system (ECF kit RPN 5752; Amersham) and following the procedure described by Kristjánsson *et al.* (1994). Increasing amounts of target DNA (25, 50 and 100 ng), denatured in

0.4 M NaOH, were slotted onto a nylon hybridization membrane (Bio-Rad) and probed with 200 ng fluorescein-labelled tracer DNA. For each duplicate of DNA–DNA association, the hybridization temperature was chosen in the optimal range in the hybridization buffer (Johnson, 1984; Ivanova *et al.*, 1988). For example, for a G+C content of 43 mol%, DNA–DNA associations were carried out for 15 h at 60 °C in buffer consisting of 4 × SSC supplemented with 19% formamide, 0.5% blocking agent, 5% dextran sulphate and 100 µg denatured sheared salmon sperm DNA ml⁻¹. Final high-stringency washes and signal amplification were performed according to the manufacturer's instructions. Hybridization signals were detected with a Storm 640 fluorescence scanner (Molecular Dynamics) and analysed with the IMAGE-QUANT program. The signal (maximum peak area) produced by hybridization of the probe with homologous target DNA was set at 100% and compared with the signal generated by heterologous DNA. DNA–DNA hybridization was also monitored by spectrophotometry (Huss *et al.*, 1983), using the same equipment as for analysing the DNA base composition. The renaturation rates were calculated using the Kontron program and the degree of annealing was estimated according to the formula of De Ley *et al.* (1970).

Fatty acid analysis. Fatty acid composition of cells grown on either *Cytophaga* marine agar or GYM (l⁻¹: 4 g glucose, 4 g yeast extract and 10 g malt extract) was determined by the DSMZ.

Cellular protein extraction and SDS-PAGE. Cell pellets were washed twice with artificial sea water, suspended in 0.5 mM Tris/HCl buffer (pH 8.0) containing 2 mM DTT, 1 mM EGTA, 1 mM EDTA and 0.4 mM PMSF, and disrupted by sonication on ice (6 pulses of 10 s at 60 W). After two runs of centrifugation at 15 000 *g* and 4 °C for 20 min, proteins were electrophoresed on a SDS-PAGE gel. Protein concentrations were estimated by the Bradford method, using a Bio-Rad assay kit. Proteins were loaded onto 12% (w/v) polyacrylamide gels (4% stacking gels) along with the following molecular mass standards: rabbit skeletal muscle myosin (200 kDa), *E. coli* β-galactosidase (116.25 kDa), rabbit muscle phosphorylase B (97.40 kDa), bovine serum albumin (66.20 kDa) and hen egg white ovalbumin (45 kDa). Gels were stained with Coomassie Brilliant Blue R250.

RESULTS

Enrichment and isolation

After 1 week of incubation of pieces of the red alga *Delesseria sanguinea* (Huds.) Lamour. at 20 °C on a basal salt medium supplemented with 2% ι-carrageenan, 14 bacterial strains were isolated. On the basis of their capability to form holes in the substratum, 7 isolates displayed agarolytic activity only and 4 isolates possessed both agarolytic and carrageenolytic activities. These latter 4 strains were pigmented yellow or orange. Based on assays for carrageenase and agarase activities (Kidby & Davidson, 1973), the isolate Dsjj^T was the most active. In the presence of 0.25% non-purified λ-carrageenan, κ- and ι-carrageenase activities were maximal (22 and 8 U ml⁻¹, respectively, at the end of the exponential phase at 20 °C; Potin *et al.*, 1991). The isolate Dsjj^T was therefore studied in more detail.

Fig. 1. ZoBell agar plate with a colony of [*Cytophaga*] *uliginosa* (on the left) and strain Dsjj^T (on the right). Plates were incubated at 22 °C for 5 d. Note the differences in colour and spreading behaviour.

Fig. 2. Phase-contrast micrograph (a) and electron micrograph (b) of strain Dsjj^T. No flagella were observed on the negatively stained cells. Bars: (a) 10 µm; (b) 1 µm.

Colonial and cellular morphology

The strain Dsjj^T exhibited spreading growth on agar plates, forming yellow–orange colonies of 1 mm diameter within 3 d at 20 °C. After 1 week of culture, the agar plate was degraded under and near the edges of the colonies. When a culture drop of the strain Dsjj^T in exponential phase was inoculated at the centre of a Petri dish, the colony expanded concentrically (Fig. 1),

degrading agar within 24 h, κ -carrageenan within 48 h and liquefying ι -carrageenan within 24 h. In comparison, carrageenase activities from [*Cytophaga*] *uliginosa* were lower, even though the agarase activities of the two strains were similar. [*Cytophaga*] *uliginosa* colonies were orange and spread at a slower rate (Fig. 1). Observations of colonies on Phytigel plates by phase-contrast microscopy indicated that both strains had a gliding motility. Strain Dsij^T was very active and cells glided towards the outside of the colony at about 1 $\mu\text{m s}^{-1}$. In comparison, [*Cytophaga*] *uliginosa* cells moved very slowly and they did not seem to leave the colony. Strain Dsij^T cells appeared as round-ended rods, about 0.3–0.4 μm wide and 3.0–8.0 μm long (Fig. 2a) and they displayed Gram-negative staining. They occurred singly or in pairs. Under the microscope, the wet mount showed non-motile cells. Non-refractile spherical bodies appeared in stationary-phase cultures. No flagella were seen by electron microscopy (Fig. 2b).

Determination of growth parameters

Strain Dsij^T grew between 13 and 45 °C, with an optimum around 35 °C, while no growth was detected at 10 or 46 °C after 15 and 3 d of incubation, respectively (Fig. 3a). In contrast, [*Cytophaga*] *uliginosa* could not grow above 42 °C. Growth was observed at NaCl concentrations ranging from 5 to 60 g l⁻¹ (Fig. 3b), with an optimum around 25 g l⁻¹ (growth in the absence of salt was observed but not after five subcultures). No growth was observed at NaCl concentrations of 70 g l⁻¹ after 3 d of incubation at 30 °C. Growth was observed between pH 6.0 and 8.5, with an optimum around pH 7.0 (Fig. 3c). No growth was detected at pH 5.5 or 9.0 after 3 d of incubation at 30 °C. Under the optimal growth conditions (35 °C, pH 7.0 and 25 g NaCl l⁻¹), the doubling time of strain Dsij^T was around 50 min.

Determination of growth requirements

Strain Dsij^T was shown to be a strictly aerobic, seawater-requiring, chemo-organotrophic and heterotrophic organism, with an oxidative metabolism that used oxygen as the electron acceptor. Nitrate could also be used as electron acceptor. The biochemical characteristics of strain Dsij^T, as compared to those of [*Cytophaga*] *uliginosa* strain DSM 2061^T, are reported in Table 1. Strain Dsij^T synthesized flexirubin and was able to degrade several polysaccharidic or proteic substrates, such as agar, κ - and ι -carrageenan, starch and gelatin. It did not display any chitinolytic activity or cellulolytic activity, either against solid (paper) or amorphous (CM-cellulose) cellulose.

Sensitivity to antibiotics

The growth of strain Dsij^T was inhibited by a concentration of 10 μg rifampicin ml⁻¹, and by 25 μg chloramphenicol or tetracycline ml⁻¹. No inhibition was observed with streptomycin, kanamycin, penicillin

Fig. 3. Effect of temperature, pH and NaCl concentration on growth of isolate Dsij^T in the *Cytophaga* marine medium described in Methods. Specific growth rates (μ) are plotted as a function of temperature in the presence of 25 g NaCl l⁻¹ at pH 7.0 (a), of the pH of the medium in the presence of 25 g NaCl l⁻¹ at 35 °C (b), and of NaCl concentration at 35 °C and at pH 7 (c).

G or penicillin A (ampicillin) at 200 $\mu\text{g ml}^{-1}$. The same results were obtained with [*Cytophaga*] *uliginosa* strain DSM 2061^T.

Table 1. Differential characteristics between strain Dsij^T and [*Cytophaga*] *uliginosa* DSM 2061^T

Determined with API 20 NE strips (identification of non-enteric Gram-negative rods), API 50 CH strips (carbohydrate assimilation and acidification), API ZYM strips (enzymic activities), and BIOLOG GN microplates (carbon substrate assimilation).

Strip	Substrate, reaction or enzyme	Dsij ^T	[<i>Cytophaga</i>] <i>uliginosa</i> DSM 2061 ^T
API 20 NE	Reduction of nitrate to nitrite	+	+
	Indole production	—	—
	Glucose fermentation	—	—
	Arginine dihydrolase	—	—
	Urea	—	—
	Aesculin hydrolysis	+	+
	Gelatin hydrolysis	+	+
	β -Galactosidase (PNPG*)	+	+
	Arabinose assimilation	±	+
	Gluconate assimilation	—	±
API 50 CH	Rhamnose assimilation	+	—
	D-Lyxose assimilation	—	±
	D-Tagatose assimilation	—	±
	2-Ketogluconate assimilation	—	+
BIOLOG GN	L-Fucose assimilation	+	—
	L-Rhamnose assimilation	+	—
	α -Ketobutyrate assimilation	—	+
	L-Serine assimilation	+	—
	L-Threonine assimilation	—	+
	Urocanic acid assimilation	—	+
	D/L- α -Glycerol phosphate assimilation	+	—
API ZYM†	Naphthol-AS-BI-phosphohydrolase	+++	+
	α -Galactosidase	+++	+
	β -Galactosidase	+	+++
	α -Fucosidase	+++	+
Starch hydrolysis	Starch agar	+	—
	Starch Phytigel	+	±
Galactan hydrolysis	Agars	+++	+++
	κ -Carrageenan	+++	+
	ι -Carrageenan	+++	+
DNA hydrolysis		+	+
Cellulose hydrolysis	Cellulose paper	—	—
	CM-cellulose	—	—

* PNPG, *p*-nitrophenyl- β -D-galactoside.

† The symbol + refers to 5 nmol hydrolysed substrate and the symbol +++ to > 40 nmol hydrolysed substrate in the API ZYM reading scale.

DNA base composition

The Chargaff's coefficient of the DNA of strain Dsij^T, as determined by the spectroscopic method (Ulitzur, 1972) and by the thermal denaturation method, were 44 ± 1 mol % ($n = 2$) and 43 ± 1 mol % ($n = 2$), respectively. The G + C content of [*Cytophaga*] *uliginosa* was 43 ± 1 mol % ($n = 2$) by the thermal denaturation

method (c.f. 42 mol % determined by Reichenbach, 1989).

16S rDNA sequence and phylogenetical analysis

The 16S rDNA sequence of strain Dsij^T was found to be closely related to [*Cytophaga*] *uliginosa*, with a sequence identity of 99.5%, the two forming a distinct

Fig. 4. Phylogenetic position of strain Dsij^T amongst some marine representatives (except *Capnocytophaga ochracea*) of the family Flavobacteriaceae. Numbers after the strain names are culture collection numbers followed by GenBank accession numbers of 16S rDNA sequences in parentheses. The topology shown is the tree obtained using the neighbour-joining method (Jukes and Cantor distance correction). Numbers at the nodes refer to the bootstrap values (100 replicates) in distance, maximum-likelihood and maximum-parsimony analyses, respectively, while dashes instead of numbers indicate that the node was not observed in the corresponding analysis. The scale bar represents the expected number of changes per sequence position.

deep branch (Fig. 4) in the family Flavobacteriaceae (Bernardet *et al.*, 1996). The other sequences in the same clade were those of *Cellulophaga baltica*, *Cellulophaga fucicola*, the *Melosira*-colonizing bacterium IC166 and *Cellulophaga lytica* (Johansen *et al.*, 1999). They displayed 91.5, 91.3, 91.3 and 90.3% sequence identity, respectively, with the 16S rDNA sequence of strain Dsij^T. Phylogenetic trees generated using distance matrix, maximum-likelihood or parsimony analyses were similar, excepting the branches with low bootstrap values or with low probabilities in the maximum-likelihood analysis (Fig. 4).

Fatty acid profile

Strain Dsij^T, [*Cytophaga*] *uliginosa* and the related species *Cellulophaga baltica*, *Cellulophaga lytica* and *Cellulophaga fucicola* exhibited similar whole-cell fatty acid profiles (Table 2), featuring three major fatty acids, 15:0 iso, 17:0 iso 3-OH and a mixture of 16:1 ω 7c and 15:0 iso 2-OH (referred to as summed feature 3 in Table 2).

DNA–DNA homology

The DNA–DNA reassociation level, as determined by slot-blot hybridization, was 46% using the DNA from Dsij^T as a probe against the DNA of [*Cytophaga*] *uliginosa* and 47% when the DNA from [*Cytophaga*] *uliginosa* was probed against Dsij^T DNA. The reassociation level, determined by the renaturation method, was 50%. These values are below the homology observed at the species level (Stackebrandt & Goebel, 1994).

Protein profiles

PAGE revealed significant differences between the protein pattern of strain Dsij^T and that of [*Cytophaga*] *uliginosa* (Fig. 5). In particular, strain Dsij^T synthesized proteins which are not present in [*Cytophaga*] *uliginosa*, such as two peptides around 97 kDa and another one with a molecular mass of approximately 66 kDa. Conversely, [*Cytophaga*] *uliginosa* produced proteins that were not present in strain Dsij^T, around 100 kDa and 110 kDa, and below 97 kDa.

Table 2. Whole-cell fatty acid profiles (percentage composition) of strain Dsij^T, [*Cytophaga*] *uliginosa* and species of the *Cellulophaga* genus

Fatty acids	Dsij ^T		[<i>Cytophaga</i>] <i>uliginosa</i>		<i>Cellulophaga lytica</i>		<i>Cellulophaga fucicola</i>		<i>Cellulophaga baltica</i>	
	Marine agar	GYM agar	Marine agar	GYM agar	Marine agar	GYM agar	Marine agar	GYM agar	Marine agar	GYM agar
13:0 anteiso								1.00		
13:1 AT 12-13	1.26	0.854		2.83	1.23					
14:0 iso								1.97		
14:0		0.98		1.32	0.84	3.09	1.00	2.54		2.57
15:1 iso G	8.49	10.86	8.68	7.48	11.64	4.13	10.46	5.40	6.22	4.91
15:1 anteiso A		1.27								
15:0 iso	23.27	18.44	38.21	24.40	20.77	21.38	23.64	18.30	11.02	9.59
15:0 anteiso	4.25	9.30		7.53	4.91	9.59	3.07	17.36		8.76
15:1 ω 6c	1.54	1.73					2.60		1.00	
15:0	7.33	6.06	10.35	8.60	5.01	2.59	9.16	3.32	9.23	6.74
16:1 iso H								0.79		3.11
16:0 iso		1.26						0.92		5.10
Summed feature 3*	11.06	13.07	9.79	11.05	4.28	7.19	8.18	13.01	14.37	20.00
16:0	2.31	4.00	3.74	7.15	1.92	8.18		2.58	5.96	4.02
15:0 iso 3-OH	5.52	5.43	5.61	3.12	11.39	10.27	14.67	10.19	4.87	5.54
15:0 2-OH					1.15			0.81		1.25
17:1 iso ω 9c	7.60	3.15	4.42	2.34	2.26		1.84	1.34		1.83
Summed feature 4†	1.36									
15:0 3-OH					1.14		2.31		0.72	
17:1 ω 8c									0.97	
17:1 ω 6c	1.00	1.06								
16:1 2-OH										2.40
16:0 iso 3-OH		1.10			6.54	6.87	2.70	5.21	3.45	8.72
16:0 3-OH	1.55	1.64	3.11	4.63	3.02	6.16	1.51	4.50	2.21	5.59
18:1 ω 5c							1.48	0.53		
17:0 iso 3-OH	14.86	11.92	16.08	11.21	15.56	16.10	10.10	8.21	9.67	7.80
17:0 2-OH		2.28		1.88	1.10			1.43		2.08

* The fatty acids 16:0 ω 7c and 15:0 iso 2-OH could not be separated from each other by GC and together were considered summed feature 3.

† The fatty acids 17:1 anteiso B and iso I could not be separated from each other by GC and together were considered summed feature 4.

DISCUSSION

The isolate Dsij^T is a Gram-negative, gliding, strictly aerobic marine bacterium capable of cleaving the red algal galactans known as agars and carrageenans. It exhibits optimal growth at 35 °C, 25 g NaCl l⁻¹ and pH 7.0, with a doubling time of 50 min. On ZoBell agar strain Dsij^T forms spreading, yellow–orange colonies.

Analysis of 16S rDNA sequences indicates that strain Dsij^T belongs to the order *Cytophagales* (CFB group or rRNA superfamily V) and to the family *Flavobacteriaceae* (Bernardet *et al.*, 1996). The G + C content of genomic DNA for strain Dsij^T is 43 mol%, within the range of this family (29–45 mol%; Bernardet *et al.*, 1996). In the 16S rDNA tree, strain Dsij^T and [*Cytophaga*] *uliginosa* form a clade with bootstrap values of 100%. This close relationship

between the two taxa was confirmed by the high score (99.5%) of sequence identity between the 16S rDNA genes of the two strains and by a high number of identical phenotypic characteristics (Table 1). Yet the differences in their total protein profiles, the low DNA–DNA hybridization values (below 50%) and the phenotypic differences between strain Dsij^T and [*Cytophaga*] *uliginosa* (Table 1) indicate that they should be recognized as two distinct species.

Although the 16S rDNA tree indicates that the Dsij^T/[*Cytophaga*] *uliginosa* branch emerges from within the clade constituted by *Cellulophaga* and the *Melosira*-colonizing bacterium, the bootstrap values of this node are 60% or less, indicating that the position of this branching is unclear. Moreover, the Dsij^T/[*Cytophaga*] *uliginosa* branch is long, showing that these two species have had a long or rapid, yet independent, evolution. Finally, the highest sequence

Fig. 5. SDS-PAGE of protein extracts from strain Dsij^T (lane 1) and [*Cytophaga*] *uliginosa* (lane 2). Protein bands were visualized by Coomassie blue staining. Arrows indicate the main differences between the two lanes. Molecular masses are indicated on the right in kDa.

identity between this branch and the genus *Cellulophaga* (91.8%, between [*Cytophaga*] *uliginosa* and *Cellulophaga baltica*) is in the same range as those observed between the genera of the *Flavobacteriaceae* (90.5–92.3%). Altogether, the phylogenetic relationships of the *Flavobacteriaceae* suggest that the [*Cytophaga*] *uliginosa*/Dsij^T clade and *Cellulophaga* constitute two distinct genera. It is also noteworthy here that both the solidity of the phylogenetic position of the *Melosira*-colonizing bacterium IC166 and its high rDNA 16S sequence identity with *Cellulophaga baltica*, 96.2%, suggest that this strain is related to the genus *Cellulophaga*.

Strain Dsij^T, [*Cytophaga*] *uliginosa* and *Cellulophaga* species all synthesize 2- and 3-hydroxylated iso/ anteiso-branched fatty acids, with chains of 15 and 17 carbon atoms (Table 2). This combination of fatty acids is characteristic of members of the *Cytophaga*/*Flavobacterium* complex. In the dendrograms inferred from these analyses, however, species clustering depended on the culture medium (data not shown), indicating that fatty acid composition is not stringent enough to discriminate the above bacteria at the genus level. In particular, *Cellulophaga baltica* markedly differed from the other *Cellulophaga* species by its low content of 15:0 iso 3-OH (Table 2).

Degradation of soluble cellulose, in the form of AZCL-hydroxyethylcellulose, was described as a character-

istic of the genus *Cellulophaga* (Johansen *et al.*, 1999). In our hands, *Cellulophaga baltica*, *Cellulophaga lytica* and *Cellulophaga fucicola* did hydrolyse CM-cellulose, whereas Dsij^T and [*Cytophaga*] *uliginosa* did not. These two strains and, more surprisingly, the three *Cellulophaga* species, did not degrade crystalline cellulose. As already observed by Johansen *et al.* (1999), other phenotypic characteristics, such as colony colour, the presence or absence of flexirubin and the G + C content of genomic DNA, as well as the maximum growth temperature separate [*Cytophaga*] *uliginosa* and Dsij^T from the genus *Cellulophaga*. [*Cytophaga*] *uliginosa* and Dsij^T are both characterized by orange colonies, a maximum growth temperature of 42 °C, the presence of flexirubin and by G + C values above 40 mol% (Table 3), whereas *Cellulophaga* species consist of yellow colonies, cannot grow above 40 °C, do not synthesize flexirubin and exhibit a Chargaff's coefficient below 33 mol% (Johansen *et al.*, 1999). These two latter features are included in the description of the genus *Cellulophaga* (Johansen *et al.*, 1999).

In conclusion, based on both the above phenotypic and phylogenetic data, we propose that the strain Dsij^T and [*Cytophaga*] *uliginosa* form a novel taxonomic group, equivalent to a new genus, *Zobellia* gen. nov., within the family *Flavobacteriaceae*, and containing two species, *Zobellia galactanovorans* gen. nov., sp. nov. and *Zobellia uliginosa* gen. nov., comb. nov. Although the genera *Flavobacterium* and *Chryseobacterium* are not closely related to *Zobellia uliginosa* and *Zobellia galactanovorans*, they also consist of yellow bacteria containing flexirubin, but they are distinguished by a low Chargaff's coefficient. Moreover, the genus *Flavobacterium* is composed of non-marine bacteria (Bernardet *et al.*, 1996) while the genus *Chryseobacterium* is characterized by the absence of gliding motility. Table 3 presents the phenotypic characteristics which distinguish these two species from the other genera of the family *Flavobacteriaceae*.

Description of *Zobellia* gen. nov.

Zobellia (Zo.bel.li.a. N.L. fem. n. *Zobellia* in honour of C. E. ZoBell, who has isolated and characterized numerous marine bacteria, notably [*Cytophaga*] *uliginosa* in 1944, and for his general contribution to the taxonomy of marine bacteria).

Marine bacteria with Gram-negative cells consisting of non-spore-forming rods (0.2 by 1.5–8.0 µm). Colonies are yellow or orange and possess a flexirubin-type pigment. Cells do not possess flagella but exhibit a gliding motility, are chemo-organotrophic and heterotrophic. Metabolism is respiratory, not fermentative and strictly aerobic, with oxygen as electron acceptor. Nitrate is reduced to nitrite. Cells hydrolyse the galactans from red seaweeds such as agar, κ-carrageenan and ι-carrageenan. Do not hydrolyse crystalline or amorphous cellulose. Produce acid from many carbohydrates. The genus belongs to the family

Table 3. Phenotypic characteristics differentiating *Zobellia* species from other member of the family *Flavobacteriaceae*

1, *Zobellia galactanovorans*; 2, *Zobellia uliginosa*; 3, genus *Cellulophaga*; 4, [*Cytophaga*] *marinoflava*; 5, *Gelidibacter algens*; 6, *Psychroserpens burtonensis*; 7, *Salegentibacter salegens*; 8, [*Cytophaga*] *latercula*; 9, genus *Psychroflexus*; 10, genus *Polaribacter*; 11, [*Flexibacter*] *maritimus* group (includes [*Flexibacter*] *maritimus* and [*Flexibacter*] *ovolyticus*); 12, genus *Flavobacterium*; 13, genus *Chryseobacterium*. —, Negative; +, positive; v, variable; O, orange; Y, yellow; R, red. Data from Wakabayashi *et al.* (1986); Reichenbach (1989); Hansen *et al.* (1992); Dobson *et al.* (1993); Vandamme *et al.* (1994); Bernardet *et al.* (1996); Bowman *et al.* (1997, 1998); Gosink *et al.* (1998); Johansen *et al.* (1999); McCammon & Bowman (2000); and this study.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Gliding motility	+	+	+	+	+	—	—	—	v	—	+	+	—
Pigment	Y–O	O	Y	Y	Y	Y	Y	O–R	O	O	Y–O	Y	Y†
Presence of flexirubin	+	+	—	—	—	—	—	—	—	—	—	v	+
Growth at 25 °C	+	+	+	+	—	—	+	+	v	—	+	+	+
Seawater requirement	+	+	+	—	+	+	—	+	v	+	+	—‡	v
Carbohydrate utilization	+	+	+	+	+	—	+	+	+	+	—	+	+
Catalase	+	+	+	+	+	+	+	—	v	+	+	+	+
Oxidase	+	+	—§	+	—	—	+	+	+	v	+	+	+
Hydrolysis of:													
Gelatin	+	+	v	NA	v	v	+	+	—¶	v	+	+	+
Aesculin	+	+	NA	+	+	—	+	NA	v	v	—**	v	+
Starch	+	—	+	+	+	—	+	—	+	+	—	v	v
Agar	+	+	+	—	—	—	—	+	—	—	—	—††	—
β-Galactosidase activity	+	+	NA	NA	—	v	+	+	—	v	—	v	v
Nitrate reduction	+	+	—	+	—	—	+	—	—	—	+	v	v
G + C (mol %)	43	42	32–33	37	36–38	27–29	37–38	32	32–36	31–34	29–32	32–37	33–38

NA, No information available.

* Gliding motility has not been observed in *Flavobacterium branchiophilum*.

† Some *Chryseobacterium meningosepticum* strains are non-pigmented.

‡ Excepting *Flavobacterium flevense*.

§ Excepting *Cellulophaga lytica*.

¶ Excepting *Flavobacterium saccharophilum*.

¶ Some *Psychroflexus gondwanensis* strains can hydrolyse gelatin.

Excepting some *Flavobacterium aquatile* strains and *Flavobacterium flevense*.

** Only reported for [*Flexibacter*] *maritimus*.

†† Excepting *Flavobacterium flevense* and *Flavobacterium saccharophilum*.

Flavobacteriaceae. The G + C content of the genomic DNA ranges from 42 to 44 mol %. The type species is *Zobellia galactanovorans*.

Description of *Zobellia galactanovorans* sp. nov.
(basonym '*Cytophaga drobachiensis*' Barbeyron *et al.* 1998)

Zobellia galactanovorans (ga.lac.ta.no.vo'rans. M.L. n. *galactan* polygalactose; L. v. *vorare* to devour; M.L. fem. adj. *galactanovorans* galactan-devouring).

Colonies on ZoBell 2216E agar plate are yellow and spread rapidly on the surface of the plate. Cells are rods (0.3–0.4 by 3.0–8.0 µm), with rounded ends and are non-motile in liquid phase. On solid surfaces, gliding motility is easily observed. Cells hydrolyse agar and κ-carrageenan. ι-Carrageenan is liquefied within 1 week at room temperature. Gelatin and DNA are also

hydrolysed. Starch is hydrolysed in starch agar and starch Phytagel plates. Cells are positive for catalase, cytochrome-c oxidase, β-glucosidase (aesculin test) and β-galactosidase activities (o- and p-nitrophenyl-β-D-galactoside tests). Cells do not produce H₂S nor indole from tryptophan. The Voges-Proskauer reaction is negative. Do not possess urease or arginine dihydrolase. D-Glucose, L-arabinose, D-mannose, D-mannitol, L-rhamnose, L-fucose, maltose, N-acetylglucosamine, D/L-glycerol phosphate or L-serine can serve as the sole carbon source, but not D-lyxose, D-tagatose, α-ketobutyric acid, L-threonine, urocanic acid, gluconate, 2-keto-gluconate, caprate, adipate, malate, citrate or phenyl acetate. Acid production is observed from D-glucose, sucrose, maltose, L-arabinose, rhamnose, D-fructose, D-galactose, D-mannose, D-mannitol and starch, but not from D-sorbitol or glycerol. The DNA G + C content is 43 mol %. The type strain is *Zobellia galactanovorans* Dsij^T, isolated

from pieces of the red alga *Delesseria sanguinea* (Huds.) Lamour. Strain Dsij^T has been deposited in the DSMZ and in the Institut Pasteur Collection under the accession numbers DSM 12802^T and CIP 106680^T, respectively.

**Description of *Zobellia uliginosa* comb. nov.
(basonym *Flavobacterium uliginosum* ZoBell and Upham 1944)**

Other synonyms: *Agarbacterium uliginosum* (ZoBell and Upham 1944) Breed 1957; *Cytophaga uliginosa* (ZoBell and Upham 1944) Reichenbach 1989.

Description was previously given for [*Cytophaga*] *uliginosa* (Reichenbach, 1989). In addition, the following features are observed. In contrast to *Zobellia galactanovorans*, colonies on ZoBell 2216E solidified with agar are orange with a very tenacious slime, without or with weak spreading activity. Moreover, the gliding motility, although easily observed, is characterized by the observation that cells do not move away from the colony. Cells do not possess an arginine dihydrolase, but feature β -glucosidase (aesculin test) and β -galactosidase (*p*-nitrophenyl galactoside test) activities. Cells hydrolyse DNA, gelatin and chitin. Starch activity is not detected on starch agar plates, but a weak activity is detected on starch Phytagel plates. Hydrolysis of κ -carrageenan and ι -carrageenan is weaker than in *Zobellia galactanovorans*, whereas both species hydrolyse agar at similar rates. D-Glucose, arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, gluconate, 2-keto-gluconate, α -ketobutyric acid, L-threonine or urocanic acid can serve as the sole carbon source, but not L-rhamnose, L-fucose, L-serine or D/L-glycerol phosphate. Acid production occurs in the presence of D-glucose, sucrose or maltose, but not in the presence of D-mannitol or glycerol.

Tables 1–3 and 2 show features that discriminate the two *Zobellia* species and distinguish the genus *Zobellia* from the other members of the *Flavobacteriaceae*.

NOTE ADDED IN PROOF

Recently, Bowman (2000) proposed that [*Cytophaga*] *uliginosa* be included in the genus *Cellulophaga*. However, as discussed above, the G + C content, maximum growth temperature, presence of flexirubin and utilization of CM-cellulose, together with the important phylogenetic distance between the genera *Cellulophaga* and *Zobellia* indicate that, in our opinion, Bowman's proposition would result in too heterogenous a *Cellulophaga* genus.

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